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Our objective is to investigate the role of cell surface integrins in metastasis of human breast cancer. Integrins play an important role in cell adhesions and interactions with extracellular matrix proteins and the maintenance of differentiation. Variants of the human breast cancer cell line MDA-MB-435 were selected for different levels of α6 integrin expression. High α6-expressing clones showed enhanced binding to laminin *in vitro*. These cells were also more metastatic to the lungs of nude mice than the clones with low expression of the integrin. The α6 integrin dimerizes with β1 in this cell line, while in the majority of human breast cancer cell lines studied α6 preferentially dimerizes with the β4 subunit. MDA-MB-435 cells lack β4 protein, possibly due to alternate splicing of β4 RNA transcript. The alternate form was sequenced and found to be novel. Both wild-type and alternate forms were seen in RT-PCR products, yet no protein was detected in FACS analyses for surface expression. Study of a panel of human breast cancer cell lines revealed a relationship between high α6 expression and malignant growth and metastasis in nude mice. The results support the hypothesis that α6 integrin expression on breast cancer cells promotes invasion and metastasis.

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The role of integrins in breast cancer metastasis

Introduction

The subject of this proposal is metastasis, the most common cause of death of women with breast cancer. At the time of diagnosis of breast cancer, prognosis is related to the disease stage and the presence of cancer cells in the axillary lymph nodes. Twenty percent of women with early stage node-negative breast cancer may subsequently develop metastatic disease, while as many as 90% of women with locally advanced breast cancer or with extensive lymph node involvement will have further or more extensive metastasis recurrence. In addition to the axillary lymph nodes, the other common sites of breast cancer metastasis are brain, liver and lungs. However, bone is the most common site of breast cancer metastasis, leading to pain, pathologic fractures, central nervous system compromise and hypercalcemia. The factors determining the pattern of metastasis of breast cancer cells to certain organs, and especially the bone have not been determined.

The objective of this proposal is to investigate the role of cell surface integrins in the metastasis of human breast cancer. Cell surface integrins play an important role in cell adhesions and interactions with extracellular matrix proteins and the maintenance of normal patterns of differentiation. Immunohistochemical analysis of breast cancer specimens has shown that alterations in integrin expression are commonly seen, with either increased, decreased or *de novo* expression of various integrins, or loss of polarization when compared with benign or normal samples. One clinical study showed that increased expression of $\alpha 6$ in primary breast cancers was related to shorter survival(1). In a nude mouse model of breast cancer metastasis developed in our laboratory(2), the cells with highest metastatic capability had higher surface expression of $\alpha 6$ and αv integrin sub-units than poorly metastatic cells. A study using the MDA-MB-435 cell line transfected with a dominant negative variant of the $\beta 4$ integrin subunit, that effectively reduced expression of the $\alpha 6\beta 1$ integrin on the cell surface showed that these cells had reduced invasive capability (3). These results complement our initial observation that the more metastatic cells had higher levels of the integrin expression, and provide further evidence for an association with malignant progression. Breast cancer cells isolated from a nude mouse bone metastasis showed elevated expression of the $\alpha v\beta 3$ integrin. This integrin is also expressed by osteoclasts and mediates binding to the bone matrix protein osteopontin (4). The bone-metastasis derived breast cancer cells bound avidly to the osteopontin, and we propose that elevated expression of the $\alpha v\beta 3$ integrin may mediate events that are critical for the development of bone metastasis.

The scope of our study is to use variants of established human breast cancer cell lines that differ in integrin expression to examine further the relationship between integrin expression and metastasis to different organs. The experimental model is the implantation of the breast cancer cells into immunodeficient nude mice. The experiments are designed to provide new information on the cancer-cell matrix interactions that are part of the metastatic progression of breast cancer. This knowledge could potentially identify new approaches for therapeutic intervention.

Task 1: Further development of an animal model for human breast cancer metastasis to bone and bone marrow

Experimental method proposed.: Injection of cells into the left ventricle of the heart of nude mice is a recognized method of inducing bone metastasis, using various types of cancer cell lines (5-7). In our preliminary studies, we showed that human breast cancer cells could be recovered from cultures of bone marrow flushed from the femurs of nude mice at intervals after injection into the left-heart.

This task has not been completed within the anticipated time frame and the experiments to complete it are still in progress. The concept is to use genetically tagged cells to examine the kinetics of bone and bone marrow metastasis development, following injection of cells into the left-heart. Tagging cells with the lac-Z gene was proposed. In the original proposal, such cells were to be obtained from another laboratory, but were not made available when requested. Transfection of cells with pCMVβ (from Clontech) was accomplished but expression of the marker gene was not stable or consistent, especially when cells were recovered from metastases in mice. As an alternative, the MDA-MB-231 and the MDA-MB-435 cell lines were transfected with the plasmid pEGFP (Clontech) expressing the green fluorescent protein, that other investigators have shown can be an effective marker in *in vivo* studies (8). In an initial test for retention of the marker gene when the breast cancer cells are injected into nude mice, MDA-MB-231-GFP cells were injected i.v. into nude mice. The numbers and incidence of lung metastases produced by the GFP-transfected cells were very similar to those produced by non-transfected cells. Cultures established from the lung metastases were examined for expression of GFP using a UV microscope, and the marker gene was still expressed. Thus this method of marking breast cancer cells appears to be stable and appropriate for further studies.

An alternative approach was to use a mouse mammary tumor line 66.3, that is highly metastatic to the lungs and lymph nodes of BALB/c mice, as a model for bone and bone marrow metastasis following injection of cells into the left heart. Other investigators using this technique report evidence of bone metastasis from paralysis or visual evidence in the mice injected with a variety of cell lines (5; 6). With the 66.3 cell line the results showed a similar distribution of metastases as we have found with human breast cancer cells injected into the left heart of nude mice. There was a high incidence of tumor nodules in the adrenals, kidney and ovaries of the mice, in addition to the lungs. Histological sectioning of the brains and vertebrae of mice is in progress, although there were no obvious symptoms of tumor development in the spine, e.g. hind quarter paralysis. While cells may be present in the bone and bone marrow, as we believe from previous work of culturing cells from bone marrow of mice injected by this route (data in proposal, and last years report), the growth of experimental metastases in other organs of the animals (e.g. kidney, adrenals) necessitates euthanasia before growth is manifest in the bone.

Task 1 progress and recommendations: Unanticipated problems with the method of tagging the breast cancer cell lines to be suitable for the study have delayed progress. The originally proposed studies, now using the GFP-tagged cells are in progress and will be completed in the next year of the project.

Task 2: Phenotypic characterization of breast cancer cells with different levels of integrin expression

a) Metastatic phenotype of MDA-MB-435 cells with different levels of α6 integrin

Experimental methods: A suspension of MDA-MB-435 human breast cancer cells was prepared by incubation with 0.02% EDTA in PBS. The cells were washed to remove excess EDTA, then suspended in PBS with 2% fetal bovine serum. Monoclonal antibody raised against human α6 integrin (Mab 1972, Centricon) was added at a 1:500 dilution and the cells were incubated on ice for 30 mins. After washing out excess antibody, the cells were then incubated with FITC-conjugated goat anti-rat IgG (Sigma Chemical Co., St Louis,MO) diluted 1:40 in PBS with 2% FBS, for 30 min on ice and in the dark. The cells were then washed with PBS and suspended at a concentration of 10^6 cells/ml. Cells expressing high α6 expression and low α6 expression were separated by analysis with an EpicsElite analyzer (Coulter, Hileah, FL), that sorted cells into the top 5% and lowest 5% on the basis of fluorescence intensity. Individual cell from the high and low expressing populations were plated in wells on 96-well microtiter plates. Clones that grew in the wells were expanded in culture and sample frozen for subsequent analyses. The expression of α6 integrin on the isolated clones was determined in the same manner as above, by incubation with specific antibodies and FITC-conjugated secondary antibodies, followed by fixation in PBS with 1% paraformaldehyde. FACS analysis was performed on an EPICS Profile Cell Sorter (Coulter) with a 525-nm band pass filter to detect FITC and gated on forward *versus* side scatter to exclude debris, dead cells and cell clumps. Analysis was based upon cursors set at 2% for isotype matched negative controls.

As a test for functional differences of α6 integrin expression between the clones, binding to laminin was tested. Microtiter plates were coated with mouse laminin (Sigma Chemical Co.) at a concentration of 20 μg/ml, 0.1 ml per well, and incubated for 16 h at 4° C. The laminin solution was aspirated and MEM with 1% BSA added, and incubated at 37°C for 1 h. MDA-MB-435 (and other variants) cells were harvested by incubation with 0.02%-EDTA and single cell suspensions prepared in MEM with 1% BSA. Cells were incubated for 30 min. with either rat IgG or α6 antibody (1:500) plated on the matrix-coated wells, or in untreated wells for the input values, and incubated for 3 h at 37°C. Non-attached cells were removed by washing with PBS, and the attached cell numbers quantified by incubation with MTT (Sigma Chemical Co.). The proportions of cells bound to the matrix-coated wells were calculated from:

$$\text{Percent bound} = \frac{\text{Absorbance from matrix bound wells}}{\text{Absorbance from input wells}} \times 100$$

For the *in vivo* study two clones were selected, one with high, and one with low α6 expression and injected into the mammary fatpad of nude mice to measure tumor growth and metastasis formation. Additional cell lines tested in the experiment were the MDA-MB-435 cells, and two variants, MDA-435Lung2 and MDA-435Br1 (9). These have high and low α6 expression respectively. Tumor growth was monitored weekly and the tumors removed surgically when of 1.5 cm diameter. All mice were killed by day 120 after injection, and the numbers of lung metastases counted.

TABLE 1- α6 AND α3 INTEGRIN EXPRESSION AND METASTASIS OF MDA-MB-435 BREAST CANCER VARIANTS

	α6		α3		Metastasis		
Cell line	%	MFU	%	MFU	%[a]	median # (range)[b]	p value[c]
MDA-MB-435	97	14.8	90	2.5	60%	5 (0 – 20)	$p < 0.001$ *
MDA-435Lung 2	100	22.6	99	21.8	90%	20 (0 – 115)	
MDA-435 Br1	68	2.4	100	26.5	10%	0 (0 – 8)	$p < 0.0001$ *
MDA-α6LF9	75	4	81	2.9	36%	0 (0 – 55)	$p = 0.011$ *
MDA-α6HG6	99	52.6	99	26.7	75%	10 (0 - >150)	$p = 1.0$ ns

a Number of mice with metastasis/number of mice with tumors x 100

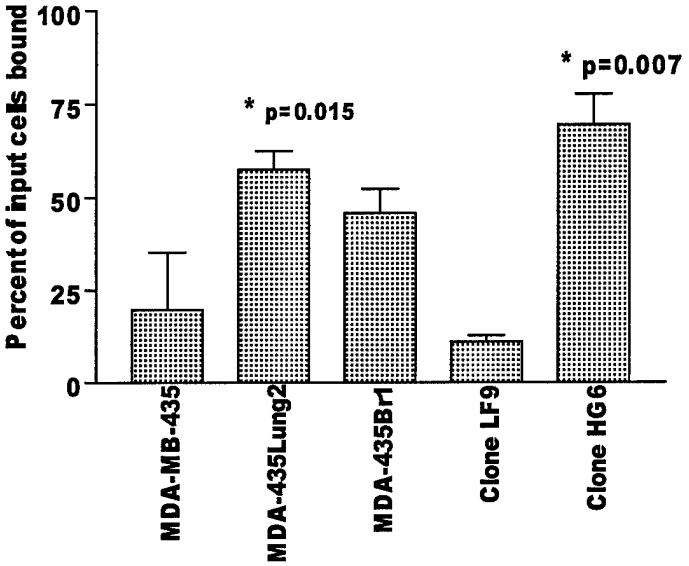
b Median number (and range) of macroscopic lung metastases

c Results of Mann-Whitney tests comparing the numbers of metastases with those in mice with the MDA-435Lung 2 tumors (ns, not significantly different). The variants with the highest expression of α6 produced significantly more lung metastases.

Results and discussion: Assays for the ability of the different clones and variants of the MDA-MB-435 cell line to bind to laminin reflected to level of expression of the α6 integrin. As shown in Fig. 1, the highest binding affinity is demonstrated by the variants with highest α6 expression, the MDA-435Lung 2 cells and the MDA-435α6HG6 cells. Binding to laminin was abrogated by the addition of blocking antibody to α6 (GoH3). The results of the FACS analysis of α6 expression on these cells are shown in Table 1.

The variants expressing the higest levels of α6 integrin produced significantly more lung metastases in nude mice that the low-expressing cells, although the growth rates of the mammary fatpad tumors did not differ (data not shown). Thus, the spontaneous metastatic potential of MDA-MB-435 breast cancer cells is associated with high levels of α6 integrin expression. High α6 expression is also correlated with binding to laminin *in vitro*, with the exception of the MDA-435Br1 cell line that has relatively low α6 expression yet binds well to the matrix protein. One explanation for this disparate finding is that the MDA-435Br1 cells express high levels of α3 integrin. This dimerizes with β1 to form a heterodimer that also binds to laminin. In this series of MDA-MB-435 variants, α3 expression corresponded with laminin binding (and antibody to α3 can block binding, though not as effectively as the antibody to α6) but the level of expression does not correlate with metastatic potential (Table 1).

Figure. 1



Task 2 progress and recommendations: The study of α6-expression related to metastatic potential is completed. A similar study using clones isolated from the MDA-MB-231 by sorting for cells with high and low αv expressing cells is in progress. Several clones have been isolated, and initial characterization by growth in agarose cultures (to test for any differences in colony forming efficiency, which can be associated with metastatic potential) and retention of

differences in αvβ3 expression is in progress. As the *in vivo* experiments proposed with the MDA-MB-231 cells (experimental metastasis) is a shorter assay than that used with the MDA-MB-435 cells lines, the study can be completed within the time frame of this award. As much of Task 3, which was scheduled for years 2 and 3 has already been accomplished (see below), there is sufficient time to complete the remaining objectives for tasks 1 and 2.

Task 3 Characterization of integrin profiles of human breast cancer cells

Experimental methods:

FACS analysis for α6 and β4 expression was as described above.
RNA extraction and RT-PCR analysis: Total RNA from the breast cancer cell lines was isolated using TRI-reagent (Sigma Chemical Co). cDNA was synthesized from 5 μg of total RNA using oligo dT as primer, then used to amplify α6, β1 or β4 PCR products with 1μM each of the 5'and 3' primers, 200 μM dNTPs and *Taq* DNA polymerase (Promega, Madison, WI). The oligonucleotides were obtained from Genosys (The Woodlands, TX). The primers for α6 mRNA were; sense 5'-CTAACGGAGTCTCACAACTC-3'; antisense 5'-ACTCTGAAATCAGTCCTCAG-3', nt 2656-3499(10). The β1 primers were; sense 5'-CAAGGTAGAAAGTCGGGACA-3'; antisense 5'-CACAGTTGTTACGGCACTCT-3', nt 2116-2454 . Two sets of β4 primers were used; Sense#1 5'-CTCAGAACACTCACACTCGA-3'; antisense#2 5'-GAGATGTGGGCCCCAGGGAG-3', nt 4679-4820; Sense #2 5'-GACGGCGGCGCGGCGGGAAGGGCGGCAGCCGTGCCCCAG-3'; antisense #2 5'-AGCTCACACTCACAAGACTC-3', nt 4441-4679 (11). Primers for β-actin were; sense 5'-GTGGGGCGCCCCAGGCACCA-3', antisense 5'-CTCCTTAATGTCACGCACGATTTC-3'. The amplified PCR products were size fractionated by electrophoresis in 4% Nusieve agarose (FMC Bioproducts, Rockland, ME) and in some instances in 2% agarose gels. The gels were stained with ethidium bromide and visualized and photographed using a UV transilluminator. PCR products were quantified by densitometric analysis and normalized with actin. The sequences of the amplified PCR products for selected samples were confirmed by DNA sequencing. Spliced variants of β4 from the MDA-MB-435 cell line was cloned into TA vector and sequenced.

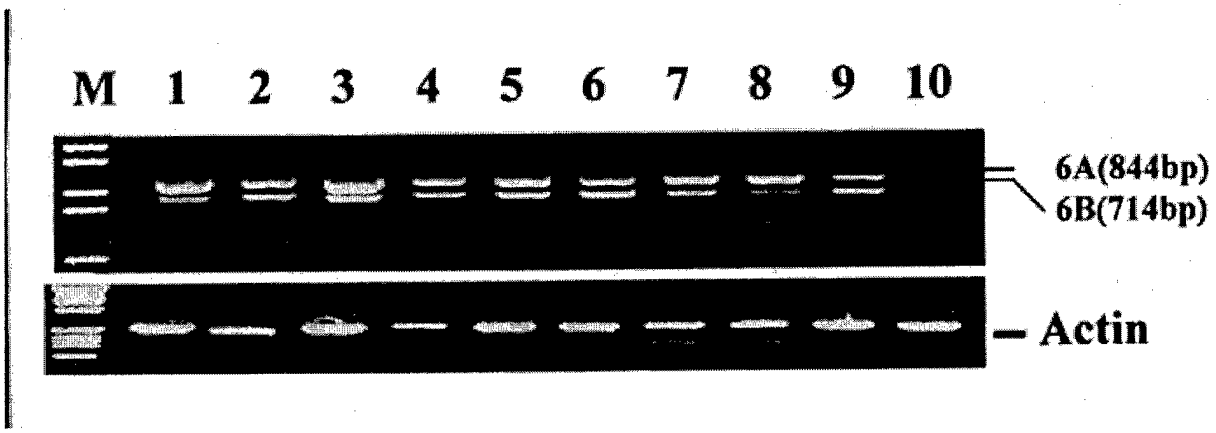
Results and discussion: The expression of α6 integrins by a panel of breast cancer cell lines was compared, using RT-PCR and FACS analysis. RT-PCR using primers described by revealed that all of the cell lines expressed the two isoforms of α6, that differ in the cytoplasmic region. With the exception of the MCF-7 cell line, the products of amplification products were at relatively equal abundance (expressed as a ratio of the β-actin product) (Fig. 2). Northern analysis, using a full-length probe of the human α6 gene (provided by Dr. Quaranta, Scripps Institute) showed that MCF-7 cells had similar levels of mRNA as the other cells lines (not shown), suggesting that the MCF-7 cells may express alternate forms, such that the product of amplification using the one set of primers did not represent the actual abundance of the gene expression. However, the cell lines differed in terms of the abundance of protein expressed at the cell surface, determined by FACS analysis (Fig. 3, Table 2). These results suggest that post-transcriptional and translational modifications in the different cell lines may determine the amounts of protein expressed at the cell surface.

TABLE 2- INTEGRIN EXPRESSION, AND TUMORIGENICITY AND METASTATIC POTENTIAL OF BREAST CANCER CELLS IN NUDE MICE

Cell Line	α6 % positive[a]	α6 MFU[b]	β4 % positive	β4 MFU	ER	Tumorigenic dose	Metastasis
MDA-MB-435	97.2	25	0	-	-	1×10^6	Yes (m.f.p.)
MDA-MB-231	98.8	30	99	58	-	2.5×10^6	Yes (i.v.)
MDA-MB-468	90.8	11.2	98.9	20.4	-	2.5×10^6	Infrequent
BT-20	92.1	13.2	100	34.2	-	5×10^6	No
BT-474	59.5	7.92	95.5	5.8	+	5×10^6	No
MDA-MB-361	59.3	7.9	84.5	7.6	+	5×10^6	No
MDA-MB-134	44.9	4.3	0	-	+	5×10^6 [c]	No
MCF-7	27.3	4.1	18.7	6.1	+	5×10^6 [c]	No

a: Percentage of cells expressing the antigen recognized by monoclonal antibody to either α6 or β4
b: Mean fluorescence intensity expressed in relative fluorescence units.
c: Dose of breast cancer cells to consistently produce tumor growth in the mammary fatpad (m.f.p.) of female nude mice.
d: Metastasis to the lungs or lymph nodes of nude mice following injection in the m.f.p. or i.v.
e Tumors grew progressively only when the mice also had s.c. implants of 17-β-estradiol.

Fig.2



RT/PCR for α6 integrin isoforms (A and B) in breast cancer cell lines. Normalized values against actin shown in figure below. Legend: M, marker; lane 1, HBL-100; lane 2, MDA-MB-134; lane 3, MDA-MB-231; lane 4, MDA-MB-361; lane 5, MDA-435; lane 6, MDA-435Lung2; lane 7,MDA-468; lane 8, BT-20; lane 9, BT-474; lane 10, MCF-7.

Figure 2b

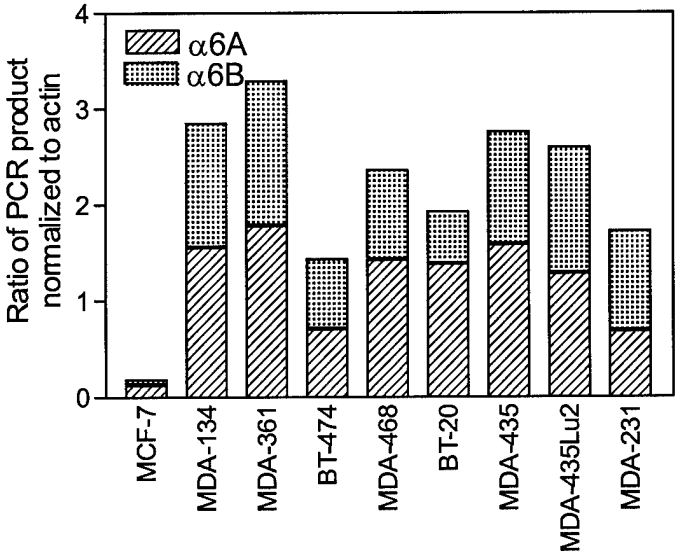


Figure 3: Expression of α6 and β4 protein in breast cancer cell lines from FACS analyses. The values plotted are the product of percent positive cells x MFU (See Table 2)

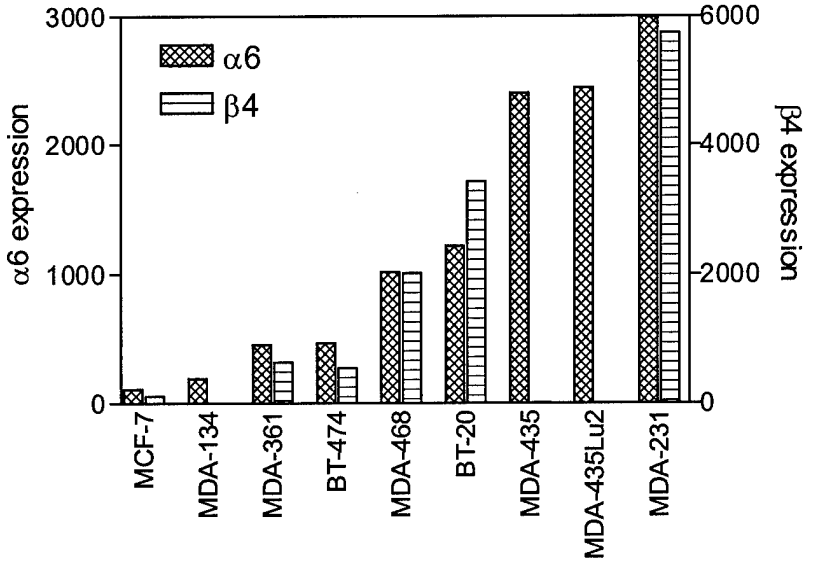
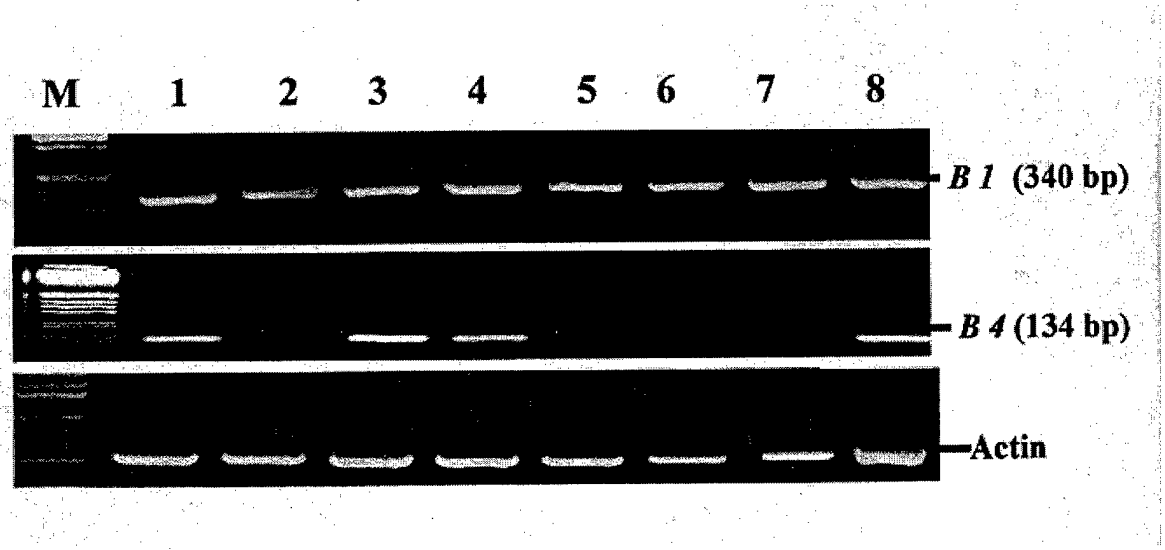


Figure 4: RT-PCR analysis of β1 and β4 integrins in human breast cancer cell lines.
Lane 1, HBL-100; lane 2, MDA-MB134; lane 3, MDA-MB-231; lane 4, MDA-MB-361; lane 5, MDA-MB-435; lane 6, MDA-435Lung2; lane 7, MDA-435Br1; lane 8, MDA-MB-468.



The inverse relationship we show between ER expression and α6 protein levels (Table 2) may suggest that some point in the regulation of α6 may be affected by steroid hormones. As discussed above, the promoter region of the α6 gene contains steroid hormone reponsive elements (12; 13; 13). In preliminary experiments, tamoxifen treatment of the ER-positive MDA-MB-134 cells reduced α6 protein levels (using a dose that over 48 h did not lead to greater than 20% growth inhibition, data not shown). No changes were seen in the amount of α3 protein expressed by control or tamoxifen treated cells, and not unexpectedly, tamoxifen had no effect on protein expression in the ER-negative MDA-MB-231 cells.

The α6 integrin dimerizes with two β subunits, β1 and β4. RT-PCR analysis revealed abundant expression of β1 in all cell lines. With the exception of two cell lines, β4 mRNA was readilt detected in all of the cell lines. The exceptions were MDA-MB-134, which showed minimal expression, and the MDA-MB-435 cells lines (and all variants derived from this cell line). In the MDA-MB-435 series a spliced variant was detected. Fig. 4 shows that a 300 bp product was amplified, in addition to the 134 bp product amplified in all other cell lines, using the primers for nt 4679-4820. The variant PCR product was subcloned into TA vector and sequenced. Homology searching in the gene bank found no match for this alternatively spliced product. Amplification using a second set of primers (more 5' than the first set) resulted in the expected product in the MDA-MB-435 samples, identical to the products from other cell lines. This result appears to confirm that the splicing event occurred with the 4679-4820 nucleotide sequence of the β4 gene.

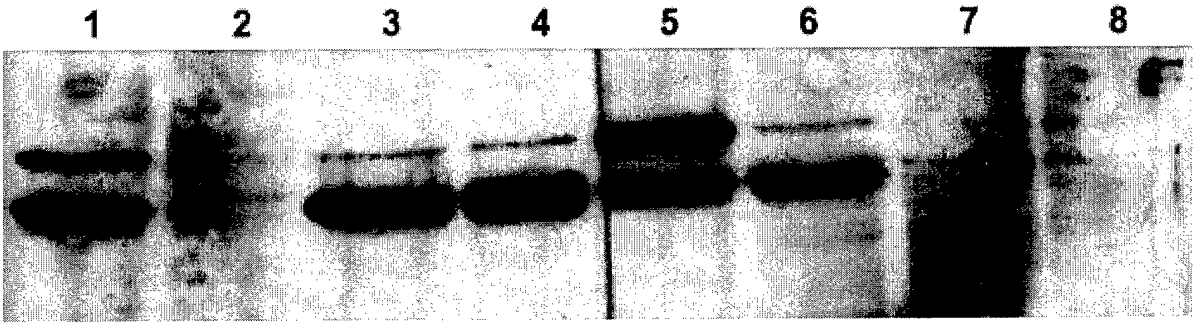
Protein expression data for β4 is shown in Table 2 and Fig. 3, and shows that the levels of this integrin sub-unit reflect the levels of expression of α6, with the exception of the two cell lines that lack full length β4 mRNA. No surface expression was detected in the MDA-MB-435 or MDA-MB-134 cell lines. Immunoprecipitation confirmed the FACS data. Precipitation with either α6 or β4 antibodies revealed similar patterns of captured proteins, showing that in the majority of the breast cancer cell lines α6 dimerized with β4. In the MDA-MB-435 and MDA-MB-134 cell line precipitation with α6 antibodies co-precipitated protein migrating at the molecular weight of β1. There are previous reports of alternate splicing of the cytoplasmic tail of β4 in different normal and malignant epithelial cells (10; 10; 11), that were compatible with protein expression. In the MDA-MB-435 cells that express the alternate form of β4, we can detect low levels of the normal transcript in RT-PCR reactions. However, the presence of the alternate form apparently eliminated translation of the β4 protein, or reduced it to below the levels of detection.

Table 2 also summarizes the results of injecting breast cancer cells into nude mice, with the minimum dose of cells that is required to produce progressively growing tumors. The table also shows the metastatic potential of the cells from either the mfp (spontaneous metastasis) or from i.v. injection (experimental metastasis). The MDA-MB-231 cell line, forms numerous lung colonies after i.v. injection of cells, although it does not metastasize from mfp tumors. In a previous report we described the MDA-MB-231 cell line as less metastastic in this experimental metastasis assay (2). Using a lower passage of the same cell line (~p120 compared with ~p490) we find that the cells will produce lung colonies at a greater efficiency (a median number of 75

lung colonies from i.v. injection of 10^6 cells, in 90 – 100% animals). The cell lines shown in Table 2 are ranked in order of the malignant growth potential in nude mice, assessing the inoculum required for progressive growth and metastatic potential. As noted above, the MCF-7 and MDA-MB-134 cell lines will only grow progressively in mice that have supplements of estrogen. Our results show a relationship between the abundance of α6 integrins on the cell surface and the malignant growth and metastatic capability in nude mice. With the exception of the two cell lines that lack abundant β4 mRNA, the level of β4 protein was similar to the level of α6 protein, with highest expression in the metastatic MDA-MB-231 cells. This result would argue against a tumor-suppressive function for β4 in human breast cancer cells. This supports the findings of Shaw *et al.* (14) who showed that introduction of normal β4 enhanced the *in vitro* invasiveness of MDA-MD-435 cells. However, our *in vivo* studies clearly show that β4 protein is not required for the invasion and metastasis of these cells. Thus our experimental finding reflect the clinical situation reported by Friedrichs e*t al.* (1) and Tagliabue *et al.*(15), of a poorer prognosis when breast cancers express high levels of α6 integrins.

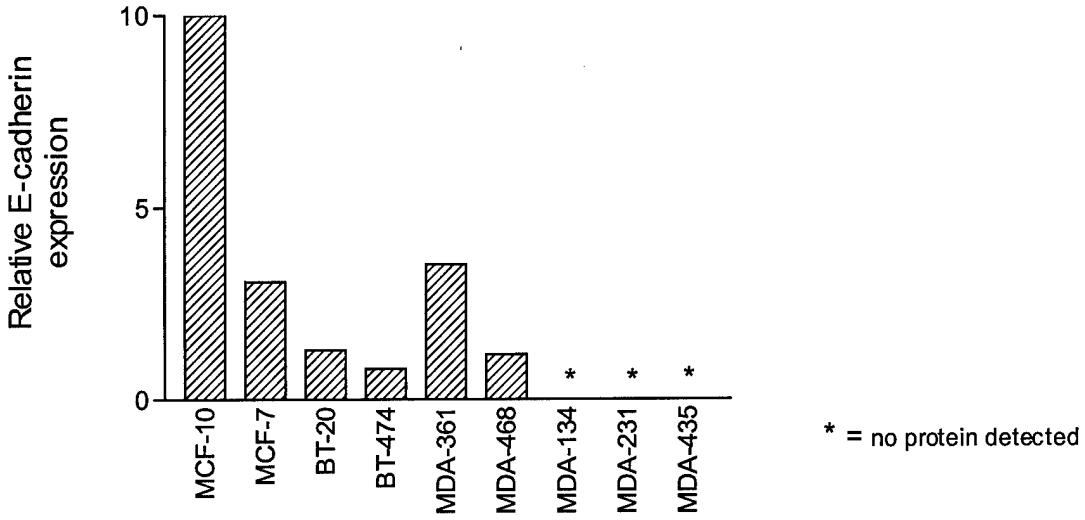
The initial impetus to investigate the role of α6 integrins in breast cancer metastasis was the clinical observations (1; 15; 15). However, the α6 integrins are obviously not the only cell surface molecules mediating cell-cell and cell-matrix interactions that can be disordered in breast cancer cells. Additional molecules are likely to contribute to the metastatic phenotype, either independently of the integrins, or potentially associated in the membrane (e.g. tetraspanin family members). For the panel of cell lines used in our preliminary studies there is a trend towards an inverse relationship between E-cadherin and α6 protein expression (Fig. 5) The one cell line that is an exception is MDA-MB-134.

Figure 5



Western blot for E-cadherin, showing the protein detected in lane 1, MCF-7; lane 2, MDA-MB-134; lane 3, MDA-MB-361; lane 4, BT-474; lane 5, BT-20; lane 6, MDA-MB-468; lane 7, MDA-MB-435; lane 8, MDA-MB-231.

When densitometric values for E-cadherin are normalized against β-actin, the results show that the highest value is in the immortalized MCF-10 cells (western image not shown), while none was detected in the two cell lines with the highest α6 expression, MDA-MB-231 and MDA-MB-435.



Task 3 progress and recommendations: objectives pertaining to α6 expression are largely accomplished. Continuing experiments with αv, and phosphorylation of $p125^{FAK}$ associated with integrins are in progress.

Task 4: Experiments are being initiated.

Conclusions: The results from Task 2 and 3 present evidence for a role of α6 integrins in the malignant progression of human breast cancer cells. More information about the regulation of expression of α6 in different breast cancer cell lines can potentially direct further studies of how to reduce expression, and hence suppress the metastatic phenotype. The highly metastatic breast cancer cell line MDA-MB-435 lacks β4 integrin expression due to an alternative splicing that eliminates expression of the protein. Within the panel of cell lines studies there was an inverse relationship between E-cadherin expression and α6 expression levels. It has been proposed that the status of E-cadherin can regulate, or is an indicator of the epithelial-medsenchyal transition of breast cancer cells. Similarly, α6 integrins may be key molecules in the transition between non-motile, differentiated cells (E-cadherin –positive, α6 low) and invasive, metastatic cells (E-cadherin –negative, α6 high), since they regulate interactions between cancer cells and the extracellular environment.

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Appendix:

Manuscript of paper submitted to International Journal of Cancer

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Appendix

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Increased levels of α6 integrins are associated with the malignant phenotype of human breast cancer cells

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Running title: α6 expression and breast cancer metastasis

SUMMARY

Integrins play an important role in interactions with the extracellular matrix, and thus have a potential role in metastasis. Expression levels of α6, β1 and β4, were measured in a panel of human breast cancer cell lines by RT/PCR, immunoprecipitation and FACS analysis. All the lines expressed α6, with the highest levels in the MDA-MB-231 and MDA-MB-435 cells. These grew the most aggressively and were metastastic in nude mice. Low levels of α6 protein were measured in breast cancer cells that were poorly tumorigenic and non-metastatic in nude mice. There was an inverse relationship between ER and α6 expression. RT/PCR revealed that all lines expressed the 2 isoforms of α6, with the α6A isoform generally more abundant than α6B isoform. Clones of MDA-MB-435 were isolated by sterile sorting for cells with high or low α6 expression, and two variants established from metastases in nude mice were found to differ in α6 expression. When injected into nude mice, the α6-high variants produced significantly more lung metastases than the α6-low variants. β1 was abundant in all lines, while β4 was not detected in MDA-MB-134 cells, and in the MDA-MB-435 variants an alternately spliced variant of β4 was identified. Sequencing of the alternate variant revealed a novel sequence from a splicing event in the cytoplasmic tail of β4. None of the cells with this variant mRNA expressed detectable levels of β4 protein. Our results suggest that high α6 expression in human breast cancer cells is associated with tumorigenicity and metastatic potential.

INTRODUCTION

The integrins are a family of heterodimeric, transmembrane glycoproteins that are formed by the non-covalent association of α and β sub-units. As transmembrane proteins the integrins can interact with extracellular molecules and intracellular proteins, interactions that define their functions. Integrins can mediate binding to various substrates, including extracellular matrix proteins, other cell surface molecules, and in some cases to other integrins. The cytoplasmic domains of integrin sub-units can engage various intracellular proteins that link up with the cytoskeleton or with adapter proteins with known signalling functions (Dedhar and Hannigan,1996; Hynes,1992; Springer,1990). At all stages in the metastatic process cancer cells interact with their immediate environment via soluble factors and contact with other cells and extracellular matrices. Altered expression of integrins mediating such interactions have been postulated to be involved in malignant cell growth, and in the process of metastasis (Giancotti and Mainiero,1994; Juliano,1994). There are a number of examples showing how introducing or upregulating integrins can increase the metastatic potential of cancer cells (Bertomeu *et al.*,1993; Chan *et al.*,1992; Matsuura *et al.*,1996). Conversely, blocking integrin expression or function using antisense constructs (Yamamoto *et al.*,1996), antibodies (Ruiz *et al.*,1993), or with competing RGD peptides can reduce metastatic potency (Saiki *et al.*,1988). The α6 integrin sub-unit dimerizes with β1 or β4 to form receptors that bind to laminin. The α6β4 heterodimer forms a component of hemidesmosomes and is important for the organization and maintenance of epithelial structures (De Melker and Sonnenberg,1996;Springer,1990). Mice lacking either α6 or β4 sub-units share the same phenotype of abherrant and blistered epithelium (Hynes,1996). Alterations in the expression of different integrins in malignant breast cells have been reported, with examples of increased, decreased, or inappropriate protein expression (Koukoulis *et*

al.,1991; Natali *et al.*,1992; Pignatelli *et al.*,1992; Zutter *et al.*,1990). Higher expression of α6 has been correlated with shorter survival in women with breast cancer. In the study by Freidrichs *et al.*(1995) 88% of the cases with distant metastases had high α6 expression in the primary tumor. Another clinical study examining both α6 and β4 expression in breast cancer biopsies also linked higher α6 expression with a greater risk of metastasis (Tagliabue *et al.*,1998). This study also reported that expression of laminin in conjunction with the integrins was indicative of poor prognosis, an observation reinforcing the concept of disordered expression of integrins and matrix by cancer cells.

In this study we sought to test the hypothesis that higher α6 expression was associated with increased malignant potential of breast cancer cells. We evaluated the expression levels of α6, β1 and β4 integrins in a panel of human breast cancer cell lines, using RT/PCR and protein measurements. The results showed that expression of these integrins is regulated at both mRNA and protein levels, and that α6 expression is inversely related to ER expression. Furthermore, that elevated levels of the α6 integrin were associated with the most aggressive malignant behavior and metastasis of MDA-MB-435 breast cancer cells as assessed in a nude mouse model.

METHODS AND MATERIALS

Cell culture: Human breast cancer cell lines were cultured in Eagle's Minimum Essential Medium supplemented with 5% fetal bovine serum (FBS), nonessential amino acids, L-glutamine, sodium pyruvate, and vitamins (GIBCO-BRL, Grand Island, NY); the supplemented medium was termed CMEM. Cell lines were maintained in monolayer culture in a humidified 37°C incubator with a 5% CO_2-95% air atmosphere.

Fluorescence-activated cell sorting (FACS) analysis: Cells grown to 70-80% confluence were harvested with trypsin and EDTA and suspended in PBS with 1% FBS at 1 x 10^6 cells per ml. Monoclonal antibodies against α6 (clone GoH3) or β4 (clone 439-9B) (Pharmingen, San Diego, CA) were added at a 1:500 dilution and incubated at 4°C for 30 minutes. The cells were then washed with PBS containing 2% FBS and 0.02% sodium azide and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (Sigma Chemical Co., St Louis, MO) at a dilution of 1:20 at 4°C for 30 minutes. The cells were washed extensively in PBS and fixed in 1% paraformaldehyde in PBS. FACS analysis was performed on an EPICS Profile Cell Sorter (Coulter, Hialeah, FL) with a 525-nm band pass filter to detect FITC and gated on forward versus side scatter to exclude debris, dead cells, and clumps. Analysis was based upon cursors set at 2% for isotype-matched negative controls. For sterile sorting of the MDA-MB-435 cell line, 5 x 10^6 cells were incubated with the α6 antibody and FITC-anti rat IgG as described above, using wash buffers without sodium azide, and the cells were resuspended after the final wash in sterile PBS. Sorting was performed on a EPICS Elite Analyzer, and approximately 5 x 10^5 cells sorted into α6-high and α6-low groups. Single cells from each group were plated into individual wells of a 96-well microtiter plate, and resulting clones expanded for subsequent studies.

Cell surface biotinylation and immunoprecipitation: Cell cultures were incubated with 0.5 mg/ml NHSLC-Biotin (Pierce, Rockford, IL) in HEPES buffer for 30 min. at 4° C, rinsed with HEPES buffer containing protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN), and the cells collected by scraping. Cell pellets were obtained by centrifugation at 500g for 5 min., then solubilized in 200 μl of the same buffer with occasional vortexing. Insoluble material was removed by centrifugation at 10,000g for 5 min. Aliquots of lysates with equal amounts of total protein were incubated overnight at 4° C with an excess of monoclonal antibody. Immune

complexes were immunoprecipitated for 1 h with 20 μl of protein A/protein G agarose (Oncogene Research Products, Cambridge MA) The bound complexes were washed and separated on 7.5% SDS-PAGE gels under reducing conditions, and transferred to nitrocellulose filters. Biotin-labeled proteins were detected using the Pierce reagent according to the manufacturer's instructions.

RNA extraction and RT-PCR analysis: Total RNA from the breast cancer cell lines was isolated using TRI-reagent (Sigma Chemical Co). cDNA was synthesized from 5 μg of total RNA using oligo dT as primer, then used to amplify α6, β1 or β4 PCR products with 1μM each of the 5'and 3' primers, 200 μM dNTPs and *Taq* DNA polymerase (Promega, Madison, WI). The oligonucleotides were obtained from Genosys (The Woodlands, TX). The primers for α6 mRNA were; sense 5'-CTAACGGAGTCTCACAACTC-3'; antisense 5'-ACTCTGAAATCAGTCCTCAG-3', nt 2656-3499 (Hogervorst et al, 1993). The β1 primers were; sense 5'-CAAGGTAGAAAGTCGGGACA-3'; antisense 5'-CACAGTTGTTACGGCACTCT-3', nt 2116-2454 (Hedman et al, 1997). Two sets of β4 primers were used; Sense#1 5'-CTCAGAACACTCACACTCGA-3'; antisense#2 5'-GAGATGTGGGCCCCAGGGAG-3', nt 4679-4820; Sense #2 5'-GACGGCGGCGCGGCGGGAAGGGCGGCAGCCGTGCCCCAG-3'; antisense #2 5'-AGCTCACACTCACAAGACTC-3', nt 4441-4679 (Tamura *et al.*,1990). Primers for β-actin were; sense 5'-GTGGGGCGCCCCAGGCACCA-3', antisense 5'-CTCCTTAATGTCACGCACGATTTC-3'. The amplified PCR products were size fractionated by electrophoresis in 4% Nusieve agarose (FMC Bioproducts, Rockland, ME) and in some instances in 2% agarose gels. The gels were stained with ethidium bromide and visualized and photographed using a UV transilluminator. PCR products were quantified by densitometric

analysis and normalized with actin. The sequences of the amplified PCR products for selected samples were confirmed by DNA sequencing. Spliced variants of β4 from the MDA-MB-435 cell line was cloned into TA vector and sequenced.

Tumorigenicity and metastasis assays in nude mice: The *in vivo* malignant properties of the human breast cancer cell lines were asessed by injection into nude mice. Tumorigenicity was evaluated by injection into the mammary fatpad (mfp), as described previously (Price *et al.*,1990). In brief, a small incision was made in the skin over the thoracic fatpad of anesthetized female mice and the mfp exposed. Tumor cells in a volume of 0.1 ml were injected into the mfp, and the incision closed with wound clips. In certain experiments, a 60-day release pellet of 0.72 mg 17-β estradiol (Innovative Research of America, Sarasota, FL) was implanted s.c. at the time of tumor cell injection. Tumor growth was monitored by weekly measurements using calipers, and tumors of 1.5 cm mean diameter removed surgically. Mice were killed 4 – 6 weeks after tumor resection. The experimental metastatic potential was assessed by i.v. injection of 10^6 cells and mice were killed up to 90 days later.

Laminin binding assays: Microtiter plates were coated with mouse laminin (Sigma Chemical Co.) at a concentration of 20 μg/ml, 0.1 ml per well, and incubated for 16 h at 4° C. The laminin solution was aspirated and MEM with 1% BSA added, and incubated at 37°C for 1 h. MDA-MB-435 (and other variants) cells were harvested by incubation with 0.02%-EDTA and single cell suspensions prepared in MEM with 1% BSA. Cells were incubated for 30 min. with either rat IgG or α6 antibody (1:500) plated on the matrix-coated wells, or in untreated wells for the input values, and incubated for 3 h at 37°C. Non-attached cells were removed by washing with PBS, and the attached cell numbers quantified by incubation with MTT (Sigma Chemical Co.). The proportions of cells bound to the matrix-coated wells were calculated from:

$$\text{Percent bound} = \frac{\text{Absorbance from matrix bound wells}}{\text{Absorbance from input wells}} \times 100$$

Statistics

The significance of differences in numbers of lung metastases was analyzed by the Mann-Whitney test. Differences in binding to laminin were analyzed using a two-sided unpaired Student's t-test.

RESULTS

Expression of α6 integrin in breast cancer cell lines: The primers used in this study to detect α6 amplified two products, representing the α6A and α6B isoforms of the integrin. All of the cell lines expressed both isoforms, albeit at different ratios; the α6A isoform is generally more abundant (Fig. 1, 2a.). Measurement of the levels of α6 protein expression, by FACS analysis, also shows that all cell lines express this integrin sub-unit (Fig. 2b, Table I). The data from the FACS analysis is expressed as the proportion of cells in the population that express the antigen, and also a relative measure of antigen density from the mean fluorescence intensity measured. The cell lines are listed in Table 1 in order of the level of expression, based on both percent positivity and the mean fluorescence intensity. A comparison of the levels of protein expression with the relative amounts of amplified products form PCR (normalized to the β-actin signal) show that there is concordance between the two assays for some cell lines (MCF-7, BT-474, MDA-MB-435) (Fig. 2). For two cell lines however, (MDA-MB-134, MDA-MB-361) the protein expression is relatively low while RT-PCR analysis revealed relatively high levels of expression. This result suggested that α6 integrin expression in the different breast cancer cell lines may be regulated at the transcriptional and post-transcriptional level.

Expression of β1 and β4 integrins: The α6 integrin dimerizes with two β-subunits, β1 and β4. RT-PCR analysis revealed abundant expression of β1 in all of the cell lines. With the exception of two cell lines, the expression of β4 mRNA was readily detected. The exceptions were MDA-MB-134 cell line, which showed minimal expression, and the MDA-MB-435 cell line (and all variants derived from this line). In the MDA-MB-435 series of cell line a spliced variant was detected. Fig. 3 shows that a 300 bp product was amplified, in addition to the 134 bp product amplified in all other cell lines, using the primers for nt 4679-4820. The variant PCR product

was subcloned into TA vector and the DNA sequenced. Homology searching in the gene bank found no match for this alternatively spliced product. Amplification using the second set of primers resulted in the expected product in the MDA-MB-435 samples, confirming that the splicing event occured within the 4679 – 4820 nucleotide sequences.

Protein expression data for β4 is shown in Table I and Fig.2b, and shows that the levels of this integrin sub-unit reflect the levels of expression of α6, with the exception of the two cell line that lack full length β4 mRNA. No surface expression was detected in the MDA-MB-435 or MDA-MB-134 cell lines. Immunoprecipitation confirmed these findings. Precipitation with either α6 or β4 antibodies revealed similar patterns of captured proteins, suggesting that in the majority of the breast cancer cell lines α6 dimerizes with β4. In the MDA-MB-134 and MDA-MB-435 cells precipitation with α6 antibodies also precipitated β1, showing that in the absence of β4 the α6 subunit dimerized with β1 (Fig. 4).

Tumorigenic and metastatic properties of the human breast cancer cell lines: Previous reports from this laboratory have shown that the mammary fatpad of nude mice is an appropriate site for assessing the tumorigenicity of human breast cancer cells (Price,1996). Table II summarizes the data from injecting breast cancer cells into nude mice, and shows the minimum dose of cells that are required to produce progressively growing tumors. The table also shows the metastatic potential of the cells, from either the mammary fatpad tumors (spontaneous metastasis) or from i.v. injection (experimental metastasis). With the exception of the HBL-100 cell line, that expresses high levels of α6 integrin (Fig. 1, and data not shown) but is not tumorigenic in nude mice, our results show that the most aggressive cell lines (MDA-MB-435, MDA-MB-231) express the highest levels of α6. The MCF-7 cell line, which is used widely as a

representative of an estrogen dependent breast cancer cell line, requires additional estrogen supplementation to support *in vivo* growth. Similarly, we have found that the MDA-MB-134 cell line will grow only in nude mice that have implants of 17β-estradiol. Even with the estrogen supplements, the minimum tumorigenic dose of cells required for progressive growth of these tumors (and others with lower α6 expression) is higher than the dose required for growth of the lines that have higher levels of α6 expression. The results show a relationship between α6 expression and malignant potential of human breast cancer cells.

Metastatic potential of selected clones and variants of MDA-MB-435: The MDA-MB-435 cell line is highly metastatic in nude mice, has high levels of α6, yet lacks β4 expression. Variants of the cell line have been isolated from metastases in nude mice, and one isolated from a brain metastasis (MDA-435Br1) was found to have reduced metastatic properties (Price *et al.* 1990). When the α6 levels were measured on this variant, lower expression was found compared with the original line, and with a variant isolated from a lung metastasis (Table III). Clones were isolated on the basis of high or low expression of α6, by sterile sorting of cells incubated with monoclonal antibody, and differences in levels of expression of the integrin were confirmed. The MDA-α6HG6 clone and MDA-435Lung 2 variant (high expression of α6) showed significantly higher binding to laminin (Fig. 6) than the original cell line and the MDA-α6LF9 (low-α6-clone). Binding to laminin was abrogated by the addition of the antibody to α6. The variants expressing highest levels of α6 integrin produced significantly more lung metastases in nude mice than the low-α6 expressing cells (Table III), although the growth rates of the different variants in the mammary fatpad did not differ (data not shown). Thus, the spontaneous metastatic potential of the MDA-MB-435 breast cancer cell line is associated with high levels of α6 expression. High α6 expression also correlated with binding affinity to laminin

in vitro, with the exception of the MDA-435Br1 cell line, which has low α6 expression yet binds well to laminin (Fig. 5). One explanation for this inconsistent finding is that the MDA-435Br1 cells express high levels of α3, which forms a dimer with β1 and mediates binding to laminin. Within the series of MDA-MB-435 variants, α3 expression apparently mediated laminin binding (antibody to α3 inhibited binding, data not shown) but the level of expression was not correlated with metastatic potential (Table III).

DISCUSSION

Altered integrin expression has been linked to the malignant phenotype of a variety of cancer types (Juliano, 1994; Giancotti and Mainiero, 1994). These observations have been interpreted as further evidence of disordered communication between malignant cells and their immediate microenvironment. Elevated expression of α6 integrins has been associated with malignant transformation of mouse skin (Tennenbaum *et al.*,1995) and the invasive and metastatic phenotypes of various rodent and human cell lines (Dedhar *et al.*,1993;Kemperman *et al.*,1993;Ruiz *et al.*,1993;Shaw *et al.*,1996;Yamamoto *et al.*,1996). This study focused on the expression of α6 integrin and associated β sub-units in established breast cancer cell lines. Our experimental results reflect the clinical findings (Friedrichs, *et al.*, 1995; Tagliabue *et al.* 1998) with the highest expression of α6 in the most aggressive cell lines, tested in a nude mouse model of breast cancer growth and metastasis.

As the different cell lines differ in many other phenotypes that might affect malignant growth potential in nude mice, we compared the α6 expression and metastatic potential of isogenic variants derived from the MDA-MB-435 breast cancer cell line. The cell line is heterogeneous for expression level of the α6 integrin, and also for metastatic phenotype.

Selection of cells from lung metastases in nude mice resulted in a cell line with high metastatic ability to the lungs of nude mice, and with high α expression. Conversely, the clone selected for high expression of the α6 integrin had high metastatic potential when injected into nude mice. Tumor growth in the mammary fatpad, and invasion through matrigel coated filters (Price *et al.*,1994) did not however differ, suggesting that the elevated expression of the α6 gave the breast cancer cells an advantage at later stages in the metastatic cascade, i.e. the arrest, extravasation and growth in the new organ (in this instance the lungs). In a previous study the high α6-expressing MDA-435Lung2 cells showed greater binding affinity to lung endothelial cells than the low α6-expressing MDA-435Br1 cells (Price *et al*, 1994). Another study using the MDA-MB-435 cell line reported that α6 expression provided a survival advantage for the metastatic cells in nude mice (Wewer *et al.*,1997). Our results concur with this previous result that α6 expression on the breast cancer cells is associated with enhanced metastatic potential.

The α6 sub-unit is preferentially expressed as a dimer with β4 in the breast cancer cell lines. β4 is known to dimerize only with α6, thus as might be expected the levels of the two integrin sub-units were generally similar in the cell lines tested. Two of the panel of cell lines lacked β4 protein expression, and in these cells α6 dimerized with β1, to form the VLA-6 laminin binding receptor. The reason for the loss of β4 expression in MDA-MB-134 cell lines is currently unknown. There are previous reports of alternative splicing of the cytoplasmic tail of β4 in normal and malignant epithelial cells (Tamura *et al.*, 1990; Hogervorst *et al.*,1990), that were compatible with protein expression. For the MDA-MB-435 breast cancer cell line we report a novel variant of the cytoplasmic region of β4. While low levels of the normal transcript were detected in RT-PCR reactions of cDNA from MDA-MB-435 variants, the presence of the

alternately spliced form presumably eliminated translation of the β4 protein, or reduced it to below the level of detection.

β4 is an unusual integrin sub-unit in that it has a long cytoplasmic domain, ~1000 aa, compared with the ~50 aa in other β subunits (Tamura *et al.*, 1990). Binding sites for adapter proteins and tyrosine phosphorylation sites have been identified in the β4 cytoplasmic domain, suggesting unique signalling functions for this integrin (Mainiero *et al.*,1995). Fibronectin like domains in the cytoplasmic tail are necessary for association with elements that form the hemidesmosomes (Spinardi *et al.*,1993), which are expressed on the basal aspect of normal mammary epithelial cells. There are conflicting reports concerning the role of β4 sub-unit in malignancy. α6β4 mediates binding to laminin-5, which is found expressed at invading fronts of some carcinomas (Pyke *et al.*,1995). The introduction of a normal β4 into a human colon cancer line and MDA-MB-435 breast cancer cells enhanced their invasion potential through Matrigel-coated filters (Shaw *et al.*,1997). Conversely, the transfection of β4 into another colon cancer cell line resulted in induction of $p21^{WAF1/CIP1}$ and growth arrest (Clarke *et al.*,1995), and complete suppression of growth of a human bladder carcinoma (Kim *et al.*,1997). In our study the level of β4, paralleling the level of α6, was highest in the more tumorigenic breast cancer cell lines, which argues against a tumor-suppressive role for β4 in the breast cancer cells. However, the results with the MDA-MB-435 line clearly show that β4 expression is not required for invasion and metastasis. One possible explanation for the apparently conflicting results in the different studies may be the potential for alternate splicing that can lead to variant forms of the cytoplasmic tail of β4 (Clarke *et al.*,1994;Strunck and Vollmer,1996;Tamura *et al.*,1990). These variants may then differ in capacity for associating with adapter proteins or other intra-cellular elements, and thus lead to different phenotypes.

The mechanisms regulating α6 expression in different cell types are currently unknown. TGF-β1 is reported to increase α6 expression, while TNF-α and IFN-γ can depress expression in various cell types (Kumar *et al.*,1995; Pirila and Heino,1996; van Valen *et al.*,1994). The inverse relationship we show between ER expression and α6 may suggest that the integrin levels are, at least in part, hormonally regulated. The promoter region of the human α6 gene has been cloned and reported by two independent groups and both report the presence of elements that are responsive to steroids (Lin *et al.*,1997; Nishida *et al.*,1997). One inference from our observation is that α6 expression may be regulated by means of steroid receptors, directly or indirectly. On going studies are testing this possibility.

An alternative explanation for our observation is that the ER-negative, high α6-expressing cells represent cells that have undergone epithelial-mesenchymal transition (Sommers *et al.*,1994). The patterns of expression seen in the breast cancer cells may be the result of co-ordinate regulation of ER and α6, and not that the integrin is modified by means of ER. Alterations in other adhesion molecules, such as E-cadherin, have been presented as examples of the shift in phenotype associated with epithelial-mesenchymal transition, which may be a critical step in the malignant progression of breast epithelium (Meiners *et al.*,1998). Whether the level of α6 expression is a marker of EMT, or is regulated by steroids, the data presented show a clear association between expression of this integrin sub-unit and the malignant potential of breast cancer cells. This reproduces the clinical situation of a poor prognosis for women with breast cancers that express high levels of α6 (Friedrichs *et al.*,1995; Tagliabue *et al.*,1998). The model of breast cancer metastasis can be used for futher analyses of how α6 integrins mediate metastasis and for the development of novel intervention strategies for treating or preventing disseminated disease.

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TABLE I EXPRESSION OF α6 AND β4 INTEGRINS BY BREAST CANCER CELLS

	α6		β4	
Cell Line	Percent positive[a]	MFU[b]	Percent positive	MFU
MDA-MB-231	98.8	30	99	58
MDA-MB-435	97.2	25	0	-
MDA-MB-468	90.8	11.2	98.9	20.4
BT-20	92.1	13.2	100	34.2
BT-474	59.5	7.92	95.5	5.8
MDA-MB-361	59.3	7.7	84.5	7.6
MDA-MB-134	44.9	4.3	0	-
MCF-7	27.3	4.1	18.7	6.1

a Percentage of cells expressing the antigen recognized by the monoclonal antibody to either α6 or B4.

b Mean fluoresence intensity expressed in relative fluorescence units.

The results shown are representative of replicate experiments.

TABLE II-TUMORIGENICITY AND METASTATIC POTENTIAL OF BREAST CANCER CELLS IN NUDE MICE

Cell Line	ER expression	Tumorigenic dose	Metastasis
MDA-MB-435	-	1×10^6	Yes (m.f.p.)
MDA-MB-231	-	2.5×10^6	Yes (i.v.)
MDA-MB-468	-	2.5×10^6	Infrequent
BT-20	-	5×10^6	No
BT-474	+	5×10^6	No
MDA-MB-361	+	5×10^6	No
MDA-MB-134	+	$5 \times 10^{6\,c}$	No
MCF-7	+	$5 \times 10^{6\,c}$	No

a: Dose of breast cancer cells to consistently produce tumor growth in the mammary fatpad (m.f.p.) of female nude mice.

b: Metastasis to the lungs or lymph nodes of nude mice following injection in the m.f.p. or i.v.

c Tumors grew progressively only when the mice also had s.c. implants of 17-β-estradiol.

TABLE III- α6 EXPRESSION AND METASTASIS OF MDA-MB-435 BREAST CANCER VARIANTS

	α6		Metastasis		
Cell line	% positive	MFU	%[a]	median # (range)[b]	p value[c]
MDA-MB-435	97	25	60%	5 (0 – 20)	$p < 0.001$
MDA-435Lung 2	99	29	90%	20 (0 – 115)	
MDA-435 Br1	68	2.4	10%	0 (0 – 8)	$p < 0.0001$
MDA-α6LF9	75	3.3	36%	0 (0 – 55)	$p = 0.011$
MDA-α6HG6	99	33	75%	10 (0 - >150)	$p = 1.0$

a Number of mice with metastasis/number of mice with tumors x 100

b Median number (and range) of macroscopic lung metastases

c Results of Mann-Whitney tests comparing the numbers of metastases with those in mice with the MDA-435Lung 2 tumors. The variants with the highest expression of α6 produced significantly more lung metastases.

FIGURE LEGENDS

Figure 1 RT-PCR of α6A and α6B isoform in human breast cancer cells. Legend: M, marker ; lane 1, HBL-100; lane 2, MDA-MB-134; lane 3, MDA-MB-231; lane 4, MDA-MB-361; lane 5, MDA-MB-435; lane 6, MDA-435Lung2; lane 7, MDA-MB-468; lane 8, BT-20; lane 9, BT-474; lane 10, MCF-7

Fig. 2 Relative abundance of α6 and β4 integrin sub-units on breast cancer cells. a) Ratio of the RT/PCR product of α6A and α6b normalized to β-actin. b) Protein expressed on the cell surface measured by FACS, plotting the product of percent positive cells x MFU (from data shown in Table I).

Fig. 3 RT-PCR analysis of β1 and β4 integrins in human breast cancer cells. Legend: M, marker lane; lane 1, HBL-100; lane 2, MDA-MB-134;, lane 3, MDA-MB-231; lane 4, MDA-MB-361; lane 5, MDA-MB-435; lane 6, MDA-435Lung2; lane 7, MDA-435Br1; lane 8, MDA-MB-468.

Fig. 4 Immunoprecipitation of α6 and β4 integrins. Legend: lane 1, MCF-7; lane 2, MDA-MB-134; lane 3, BT-474; lane 4, MDA-MB-361; lane 5, MDA-MB-468; lane 6, MDA-MB-435; lane 7, MDA-MB-231.

Fig. 5 *In vitro* binding of MDA-MB-435 cells to laminin. The percentages of the input number of cells attached after 3 h incubation on laminin-coated surfaces are shown. Significantly more MDA-435Lung 2 and MDA-α6HG6 cells bound to laminin compared with the original MDA-MB-435 cell line (Student's t-test).

Figure 1

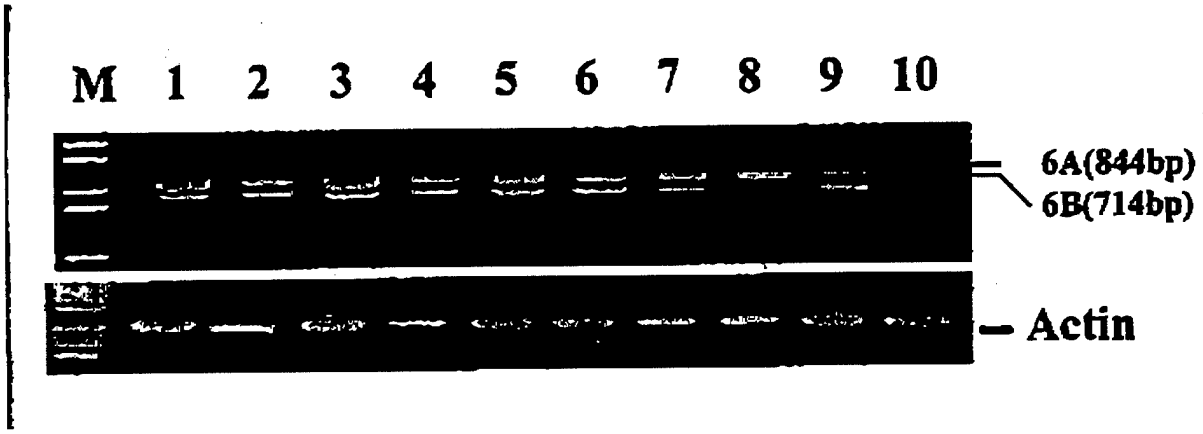


Figure 2a

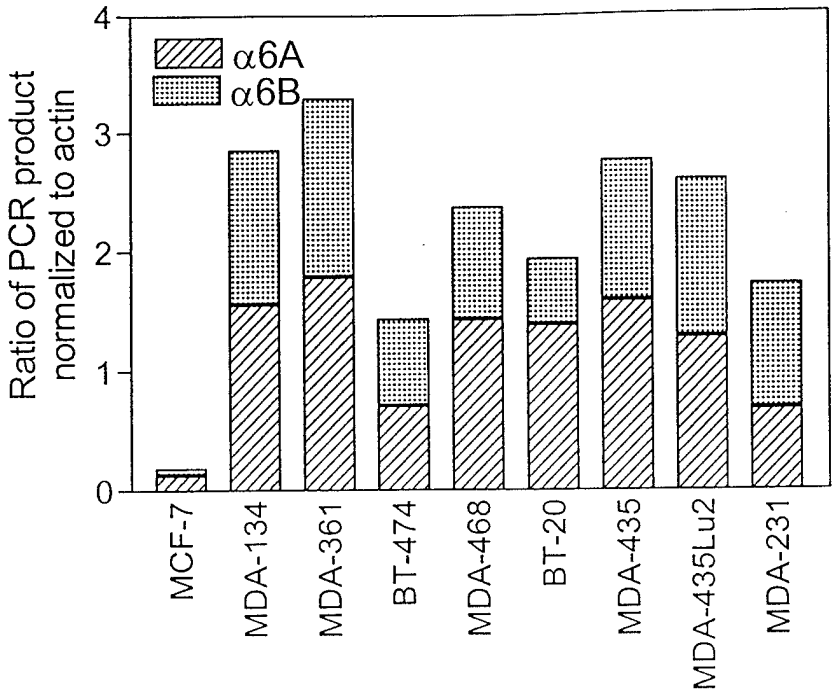


Figure 2b

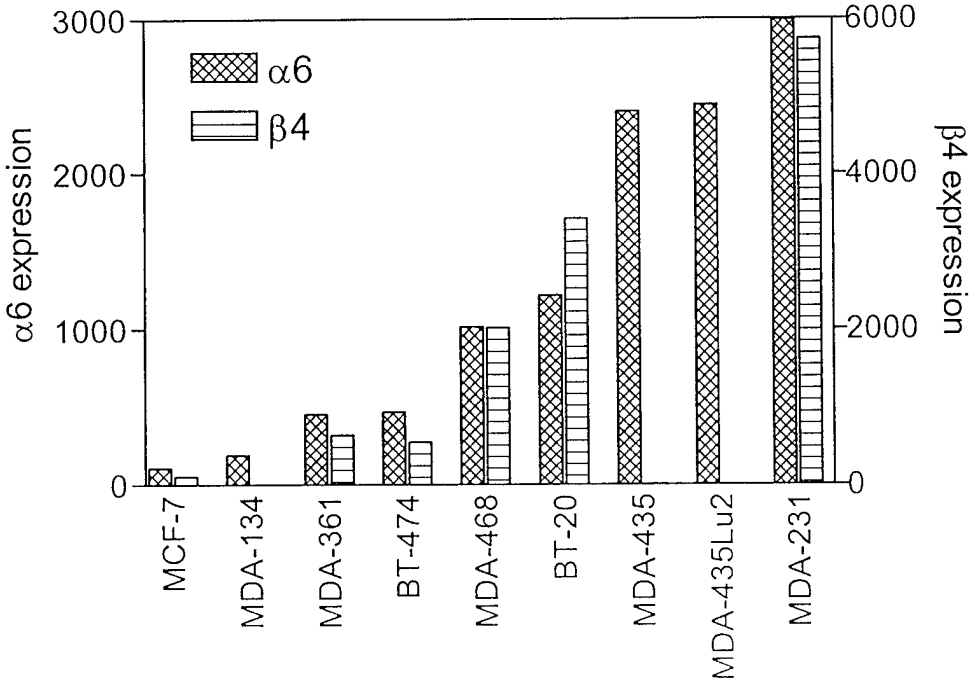


Figure 3

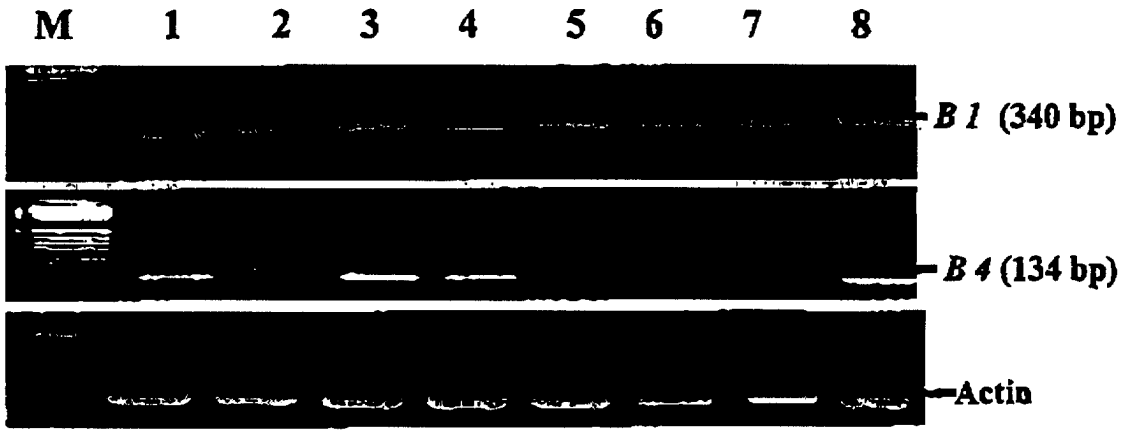


Figure 4

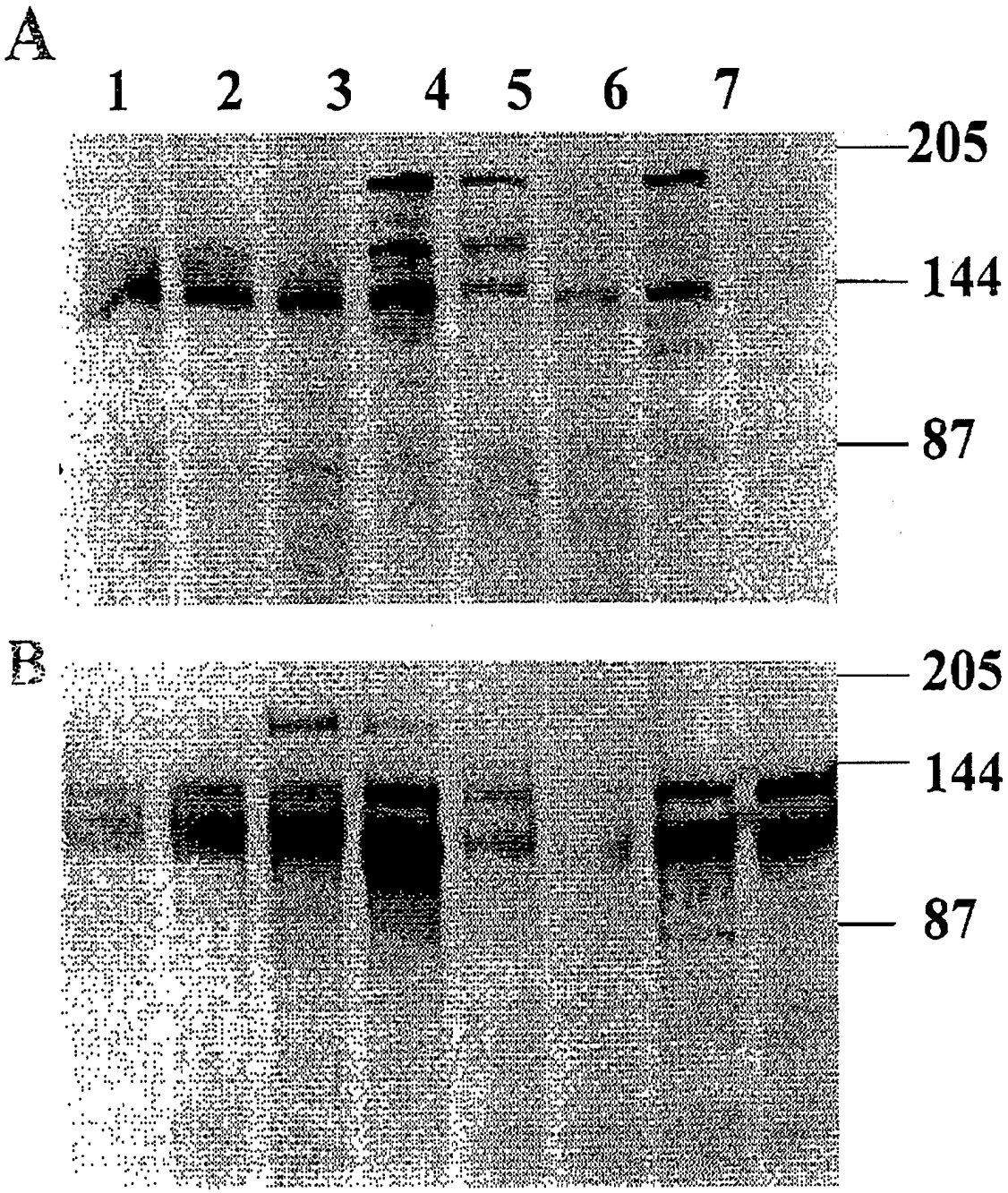
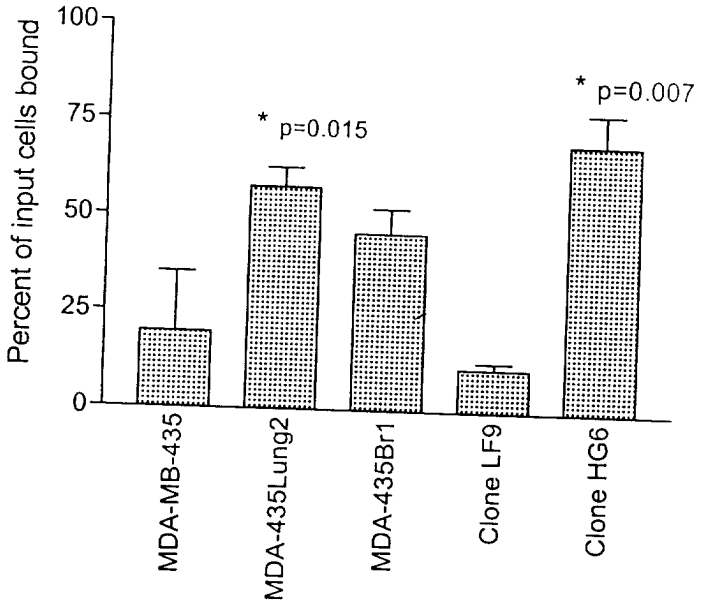


Figure 5


Percent of input cells bound
100
75
50
25
0
* p=0.015
* p=0.007
MDA-MB-435
MDA-435Lung2
MDA-435Br1
Clone LF9
Clone HG6